Effect of Storage Temperature and Duration on the Behavior of *Escherichia coli* O157:H7 on Packaged Fresh-Cut Salad Containing Romaine and Iceberg Lettuce

Yaguang Luo, Qiang He, and James L. McEvoy

Abstract: This study investigated the impact of storage temperature and duration on the fate of *Escherichia coli* O157:H7 on commercially packaged lettuce salads, and on product quality. Fresh-cut Romaine and Iceberg lettuce salads of different commercial brands were obtained from both retail and wholesale stores. The packages were cut open at one end, the lettuce salad inoculated with *E. coli* O157:H7 via a fine mist spray, and resealed with or without an initial N_2 flush to match the original package atmospheric levels. The products were stored at 5 and 12 °C until their labeled "Best If Used By" dates, and the microbial counts and product quality were monitored periodically. The results indicate that storage at 5 °C allowed *E. coli* O157:H7 to survive, but limited its growth, whereas storage at 12 °C facilitated the proliferation of *E. coli* O157:H7. There was more than 2.0 log CFU/g increase in *E. coli* O157:H7 populations on lettuce when held at 12 °C for 3 d, followed by additional growth during the remainder of the storage period. Although there was eventually a significant decline in visual quality of lettuce held at 12 °C, the quality of this lettuce was still fully acceptable when *E. coli* O157:H7 growth reached a statistically significant level. Therefore, maintaining fresh-cut products at 5 °C or below is critical for reducing the food safety risks as *E. coli* O157:H7 grows at a rapid, temperature-dependent rate prior to significant quality deterioration.

Keywords: *E. coli* O157:H7, fresh-cut, lettuce, quality, temperature

Practical Application: Specific information regarding the effect of temperature on pathogen growth on leafy greens is needed to develop science-based food safety guidelines and practices by the regulatory agencies and produce industry. Temperature control is commonly thought to promote quality of leafy greens, not safety, based at least partially on a theory that product quality deterioration precedes pathogen growth at elevated temperatures. This prevalent attitude results in temperature abuse incidents being frequently overlooked in the supply chain. This study demonstrates that human pathogens, such as *E. coli* O157:H7, can grow significantly on commercially packaged lettuce salads while the product's visual quality is fully acceptable. Packaged fresh-cut salads are marketed as "ready-to-eat" while lacking an effective pathogen kill step during their preparation. Thus, maintaining storage temperature at 5 °C or below is critical to prevent pathogen proliferation and mitigate food safety risks should pathogen contamination inadvertently occur during crop growth or postharvest fresh-cut processing.

Introduction

Packaged fresh-cut vegetables are popular food products, because they offer convenience in products with freshness and high nutritional value. However, the cut vegetable tissues provide substrates and environmental conditions conducive to the survival and growth of various microorganisms, including foodborne pathogens such as *Escherichia coli* O157:H7 and *Listeria monocytogenes* (Abdul-Raouf and others 1993; Delaquis and others 2007; McEvoy and others 2008). Between 1995 and 2006, more than 20 outbreaks of *E. coli* O157:H7-associated illnesses were linked to fresh-cut lettuce and spinach in the United States (CDHS 2007; Herman and others 2008; Lynch and others 2009). Pathogens may contaminate fresh produce in the field or at any point in the supply chain (Beuchat 2004; Brackett 2005). Thus, maintaining the safety of fresh-cut produce is of particular concern and remains a major challenge to the industry and the consumers.

Many washing and sanitizing methods have been reported for the antimicrobial treatment of fresh-cut produce (Gonzalez and others 2004; Luo 2007; Allende and others 2008). Although sanitizers can reduce microbial populations, none of the available methods can be relied upon to eliminate foodborne pathogens. The contaminating pathogens are not all accessible to a

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sanitizer-containing aqueous solution because of the presence of hydrophobic regions, fissures, cavities, and rough surfaces of vegetables, which may harbor pathogens (Zhou and others 2009). In addition, studies have documented that *E. coli* O157:H7 can penetrate into the stomata and junction zones of cut lettuce, thus becoming protected from sanitizer treatment (Takeuchi and Frank 2000). Therefore, preventing pathogen contamination and limiting its growth are critical measures to ensure the safety of fresh-cut produce, especially since fresh-cut vegetables are marketed as ready-to-eat without a further microbial kill step.

Storage temperature is known to be the single most important factor affecting the quality of fresh-cut vegetables (Jacxsens and others 2002; Izumi and others 2005). Produce maintains fresher appearance and lasts longer when temperature control is adequate (4 °C or less), while storage at abusive temperature can facilitate rapid quality decline as a result of proliferation of spoilage bacteria (Smyth and others 1998; Kader 2002). There is evidence that the growth of *E. coli* O157:H7 is inhibited at temperatures of 4 °C or less (Francis and O'Beirne 2001) whereas *E. coli* O157:H7 is capable of growing at 8 to 12 °C (Francis and O'Beirne 1999, 2001; Li and others 2001), the temperature range that fresh-cut produce is frequently exposed to during marketing and distribution (Jol and others 2005). This study addressed the question: can packaged fresh-cut salads from different brands support *E. coli* O157:H7 growth under situations of temperature abuse; and can proliferation of *E. coli* O157:H7 populations on produce occur prior to obvious degradation of visual quality? Visually enticing contaminated fresh-cut products are more likely to be consumed than those with obvious signs of decay.

While our earlier studies evaluated the effect of storage temperature regimes on pathogen growth on baby spinach leaves (Luo and others 2009), the main objective of this study was to investigate the effect of temperature control on the behavior of *E. coli* O157:H7 on packaged fresh-cut lettuce salads in relation to product quality deterioration. Fresh-cut Romaine and Iceberg lettuce were the focus of this study as they are the major ingredients of many packaged salads.

Materials and Methods

Preparation of *E. coli* O157:H7 inoculum

Lettuce outbreak *E. coli* O157:H7 strain F6460 was obtained from CDC and adapted for nalidixic acid (Nal) resistance according to Wachtel and Charkowski (2002). In brief, *E. coli* strain F6460 overnight culture grown in Luria-Bertani (LB) broth (Difco Laboratories, Detroit, Mich., U.S.A.) at 37 °C was plated on LB agar supplemented with nalidixic acid. Spontaneous nalidixic acid-resistant mutants were isolated following 3 consecutive transfers on LB-Nal and were maintained at −80 °C in LB containing 25% (v/v) glycerol (Wachtel and others 2003). Prior to using F6460-Nal^R for evaluating temperature effect on *E. coli* O157:H7 survival and growth on leafy green vegetables, the growth patterns of strain F6464-Nal^R and F6464 on lettuce and spinach extracts, and their response to storage temperature were examined. No significant difference was found between F6460 and F6460-Nal^R strains (data not shown).

To prepare the inoculum, the *E. coli* O157:H7 F6460-Nal^R strain was streaked out from the stock culture and grown overnight in LB broth at 37 °C. The culture was then transferred to nalidixic acid (25 μg/mL) supplemented LB broth and again grown overnight at 37 °C. An initial inoculation level of approximately 10^3 CFU/ g applied to salads in trials 1 and 2 was obtained by diluting the overnight culture in sterile PBS. For experimental trial 3, the cells were stored at 5 °C prior to using as an inoculum to simulate cold and nutrient stress, the physiological condition of *E. coli* O157:H7 that may be found in the produce field. The bacterial cells were then diluted and inoculated onto salads as in trials 1 and 2.

Procedures for inoculation and treatment

For trials 1 and 2, commercially packaged salads containing primarily Romaine and Iceberg lettuce were ordered and purchased upon arrival from local retail establishments. For trial 3, recently delivered commercially packaged fresh-cut salads were obtained from a local distribution center. The bagged salads were transported to the Produce Quality and Safety Laboratory (Beltsville, Md., U.S.A.) and stored at 5 °C until inoculation, which was carried out within 2 h. The inoculation of packaged lettuce salads with *E. coli* O157:H7 was conducted by spraying diluted inoculum preparation directly into the packages. The inoculum preparation was transferred into a small vial equipped with an atomizer. One corner of the package was cut to allow the spray nozzle of the atomizer to be inserted into the package. Fine mists of *E. coli* O157:H7 inoculum were sprayed onto the lettuce. The corner of the package was then sealed hermetically via an impulse heat sealer (Model PFS-F450, Kingstar Group, Wenzhou Zhejiang, China). The same procedure was repeated 4 times for all 4 corners of the package to ensure uniformity of the inoculation. Each spray was quantified (0.125 mL/spray) and a total of 8 sprays (1.0 mL) were used per package (360 g). The initial inoculum concentration on lettuce was confirmed by plating on Sorbitol MacConkey agar supplemented with nalidixic acid (25 μg/mL) (SMACN) and incubating at 37 °C for 16 to 24 h. In trials 1 and 2, packages were resealed after inoculation without removing any lettuce. For trial 3, a fixed amount (6 g) of lettuce was removed to compensate for the reduction in package size caused by resealing the packages. Packages were flushed with N_2 until a desired O_2 level was reached (approximately 1.3 kPa O_2) before sealing to simulate the commercial practice to prevent lettuce browning. For all trials, packages were manually shaken to assist inoculum distribution after sealing. Packages were then promptly transferred to 5 or 12 °C cold rooms and stored until the expiration of the "Best If Used By Date" (10 d postinoculation). The entire experiment was carried out in a Biosafety Level 2 Laboratory.

Enumeration of *E. coli* O157:H7

Populations of *E. coli* O157:H7 were enumerated on inoculated lettuce salads stored at 5 and 12 °C. Randomly sampled lettuce (25 g) was taken from each package, placed into sterile filter stomacher bags, and macerated in sterile PBS with a stomacher blender (Seward Stomacher 400, London, U.K.) at 230 rpm for 2 min. A Spiral Plate System (Autospiral^{TM} DW, Don Whitley Science Ltd., West Yorkshire, U.K.) was used to logarithmically spread the filtrates or their appropriate dilutions onto SMACN supplemented with sodium pyruvate (0.1%, w/v). After incubating plates at 37 °C for 16 to 24 h, the *E. coli* O157:H7 colonies were enumerated with a Protocol Colony counter (Model 5000, Synoptics Ltd., Cambridge, U.K.). Serological testing using a rapid RIM^{TM} *E. coli* O157:H7 latex agglutination assay (Remel Inc., Lenexa, Kans., U.S.A.) further confirmed sample *E. coli* O157:H7 colonies.

Package atmosphere determination and product quality assessment

On the day of sampling, a 10-mL gas sample was removed from each package headspace and passed through a sterile filter

(0.22 μm) with a gas-tight syringe. The sterilized gas sample was then injected into a CO_2/O_2 gas analyzer (Oxygen, Model S-3A/I, Sensor N-22M; Carbon Dioxide, Model CD-3A, Sensor P-61B; Ametek, Pittsburgh, Pa., U.S.A.) for analysis.

The overall visual quality (OVQ) of the packaged lettuce salad samples was assessed by 6 members of an expert sensory panel who rated the product based on a 9-point hedonic scale where 9 = like extremely; 7 = like moderately; 5 = neither like nor dislike; 3 = dislike moderately; and 1 = dislike extremely (Meilgaard and others 1991; Lopez-Galvez and others 1997; Luo 2007). The OVQ assessments were conducted before and after opening the packages. For the after-opening assessments, salads were first sampled for subsequent enumeration of *E. coli* O157:H7 and the rest of the samples were then coded with random 3-digit numbers to mask the treatment identity to minimize subjectivity and ensure the assessment accuracy.

Experimental design and statistical analysis

The experiment was conducted using a completely randomized block design with day 0 samples treated as detached controls. Experimental units were bags and there were 4 replications per treatment per evaluation period. Data were analyzed as a 2-factor linear model using the PROC MIXED procedure (SAS Inst. Inc., Cary, N.C., U.S.A.) with storage time and temperature as the factors. Normality and variance homogeneity of the linear model were checked for the log-transformed data. A variance grouping technique was used to address variance heterogeneity for means comparisons. When effects were statistically significant, mean comparisons were done with Sidak adjusted P values to maintain experiment-wise error ≤ 0.05.

Results and Discussion

Changes in *E. coli* O157:H7 populations

Figure 1 and 2 display the changes in *E. coli* O157:H7 populations on salad processed from 2 leading commercial brands A and B. In trial 1, when held at 12 °C, *E. coli* O157:H7 populations increased significantly ($P < 0.0001$) over time on samples from brand A; however, although noticeable, the growth of *E. coli* O157:H7 over time on samples from brand B did not reach

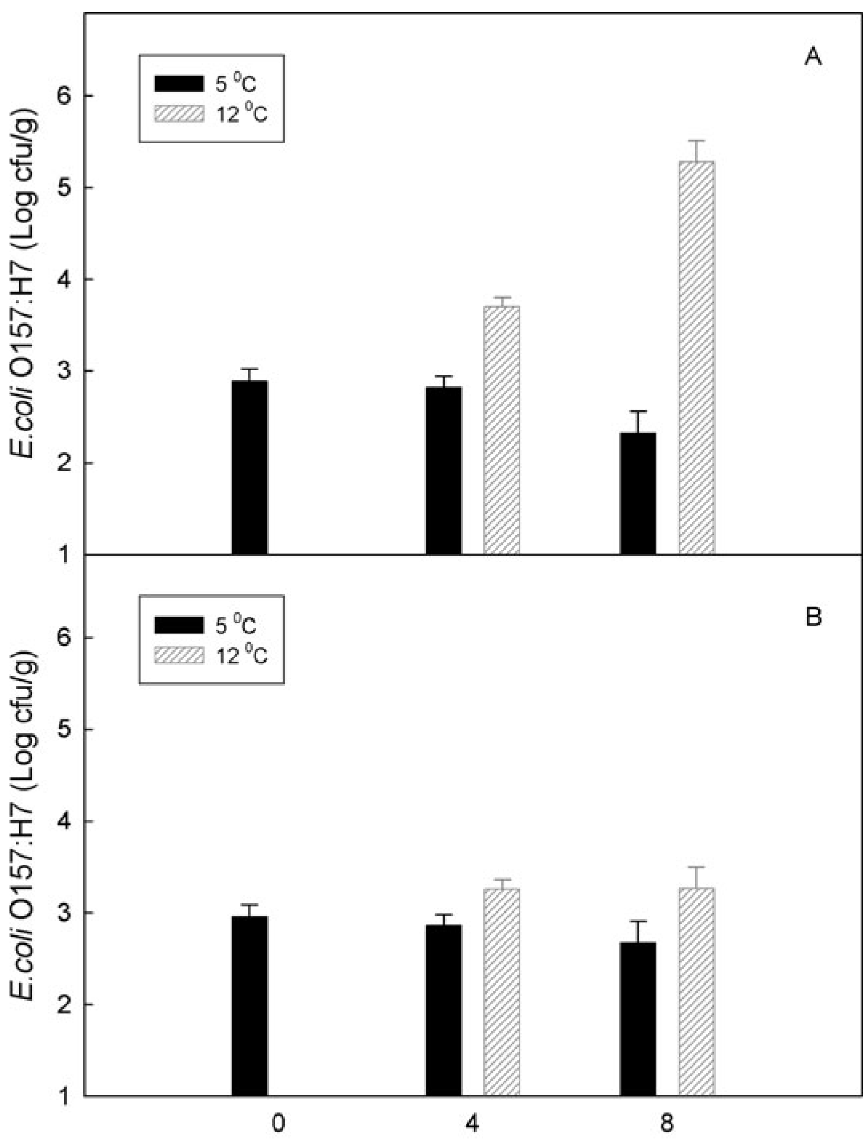

Figure 1–Trial 1-changes in *E. coli* O157:H7 populations on fresh-cut lettuce, stored at 5 and 12 °C, of 2 commercial brands of A and B. Reported populations represent the means of 4 replications. The vertical bars represent the standard errors.

a statistically significant level ($P > 0.05$) (Figure 1). When held at 5 °C, no significant ($P > 0.05$) growth of *E. coli* O157:H7 was found on samples from either brand A or B for the entire storage duration. In trial 2, *E. coli* O157:H7 grew significantly ($P < 0.001$) on samples from both brands A and B when held at 12 °C (Figure 2), but the increase in *E. coli* O157:H7 was more pronounced on samples from brand B than on samples from brand A. As shown in Figure 2B, *E. coli* O157:H7 populations on brand B increased from 2.9 log CFU/g on day 0 to 4.7 log CFU/g on day 5, and 6.5 log CFU/g on day 8 when held at 12 °C; whereas *E. coli* O157:H7 on brand A grew from 2.9 to 3.7 log CFU/g on day 5, to 5.3 log CFU/g on day 8 when held at 12 °C (Figure 2A). When held at 5 °C, similar to the results from trial 1, there was no growth, or indeed a slight decline in *E. coli* O157:H7 populations on samples of both brands A and B during storage. These findings suggest that there was no significant difference between samples of the 2 tested commercial brands in terms of *E. coli* O157:H7 growth under temperature abuse situation. However, the growth of *E. coli* O157:H7 was affected by the lettuce physiological and microbiological conditions at the time of inoculation, and perhaps the competition between *E. coli* O157:H7 and natural microflora. In trials 1 and 2, the commercially packaged lettuce salads were cut open, inoculated, and then resealed. In addition to storage temperature, 2 other environmental factors may have also affected product quality and *E. coli* O157:H7 growth. First, since there was no gas flush during the resealing of the packages, the initial package atmospheric composition had an initially higher O_2 and lower CO_2 than that of the commercial packages at the time of the inoculation (prior to opening the bags). Second, the resealing of the packages resulted in a reduced package size, and thus significantly decreased the total respiring surface of the package. Since package atmospheres at equilibrium are dependent upon the total respiration surface, the oxygen transmission rate of the package film, product weight, and respiration rate (Luo and others 2004; Kim and others 2005), the significant reduction in package size resulted in lower than optimal O_2

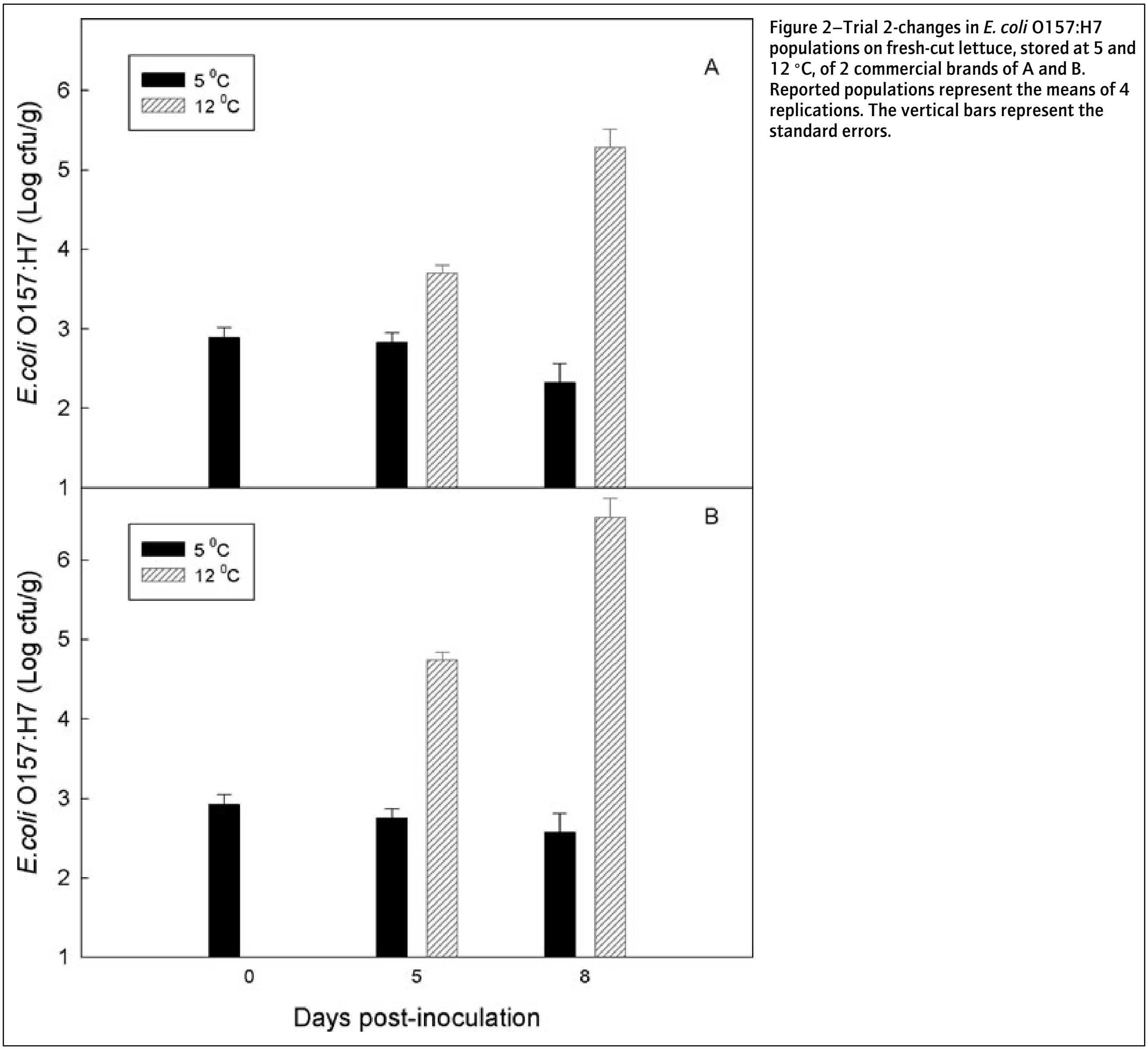

Figure 2–Trial 2-changes in *E. coli* O157:H7 populations on fresh-cut lettuce, stored at 5 and 12 °C, of 2 commercial brands of A and B. Reported populations represent the means of 4 replications. The vertical bars represent the standard errors.

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and higher than optimal CO_2 levels in the equilibrium package atmosphere. The high initial O_2 content after inoculation accelerated browning of the lettuce, while the low O_2 and high CO_2 at equilibrium resulted in lettuce tissue physiological disorders, decay development, and the accelerated growth of lactic acid bacteria and decay when held at abusive temperature. The measured package atmospheres are consistent with our visual observations that all samples held at 12 °C became unacceptable after 4 to 5 d of storage. For samples obtained from brand B in trial 1, it was noted that the lettuce was in a poor condition at the time of inoculation, probably caused either by temperature abuse prior to our obtaining the samples, or poor raw product quality at the time of processing.

To overcome the issues associated with less than optimal quality of product obtained from retail storage, the reduction in package size due to resealing and lack of initial oxygen control, a 3rd trial was conducted. In this trial, packaged lettuce was obtained soon after processing upon its arrival at a national distribution center. A set amount (6 g) of lettuce was removed to adjust for the total respiration surface reduction during resealing. The package atmospheres were tested prior to opening the bags, and the bags were flushed with N_2 before resealing to attain O_2 levels that matched the original. Furthermore, since *E. coli* O157:H7 cells that exist in the produce field may be cold and nutrient stressed, *E. coli* O157:H7 inocula were preconditioned at 5 °C for 48 h to mimic this real life situation. As shown in Figure 3, the initial inoculation level of *E. coli* O157:H7 on lettuce was 2.7 log CFU/g. *E. coli* O157:H7 grew significantly over time on lettuce stored at 12 °C and the population reached 5.0 log CFU/g on day 3, 5.5 log CFU/g on day 5, and 6.3 log CFU/g on day 7, respectively. On the contrary, *E. coli* O157:H7 populations decreased over time during 5 °C storage, with the average population declining to 1.9 log CFU/g by day 10.

Changes in package atmospheres

The atmospheric composition of the salad package headspace was tested before opening and packages were flushed with N_2 to match the package atmospheres in the commercial products before resealing after inoculation. After the flushing treatment, the O_2 and CO_2 concentrations in the lettuce packages were 1.27 and 1.51 kPa, respectively (Figure 4). Storage temperature significantly affected package atmosphere composition at equilibrium. During storage at 5 °C, there was a gradual decrease in O_2 and increase in CO_2 in the packages, with O_2 ranging from at 0.25 to 0.7 kPa after 3 d of storage, and CO_2 reaching 12 kPa by the end of storage. While following the same trends, the changes in both CO_2 and O_2 were more rapid and significantly more pronounced in products held at 12 °C than those at 5 °C; the O_2 levels in the lettuce packages at 12 °C, averaged 0.2 kPA after 3 d of storage and CO_2 increased to 19.39 kPa by the end of storage. Changes in package atmospheres of inoculated samples matched well with those un-inoculated and un-opened samples, suggesting that the procedures used in trial 3 (removal of a portion of lettuce samples to compensate for the package size reduction, initial N_2 flush, and so on) were able to create package atmospheric conditions, after inoculation, that mimic the commercially packaged samples used in the experimental trial.

Visual quality

On each sampling date of trial 3, the overall visual qualities of packaged fresh-cut products were assessed both before and after opening the bags as the visual qualities of these 2 conditions often influence the decision of purchasing and consumption, respectively. Although the quality of the packaged salads declined over time during storage, the quality remained high for the first 3 d at both 5 and 12 °C (Figure 5). The quality of products declined significantly more at 12 °C than at 5 °C. When stored

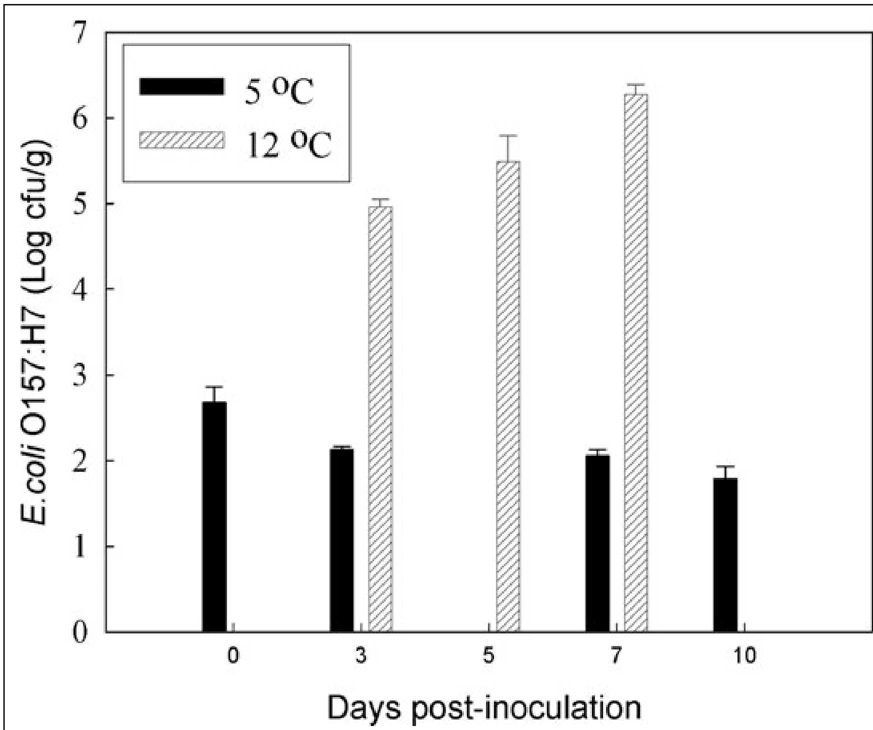

Figure 3–Trial 3-changes in population of *E. coli* O157:H7 on fresh-cut lettuce stored at 5 and 12 °C, for up to 10 d. Reported populations represent the means of 4 replications. The vertical bars represent the standard errors. Samples stored at 12 °C were collected more frequently, with a shorter duration, than those stored at 5 °C.

at 5 °C, products maintained acceptable quality throughout the entire storage period. On day 7, the quality of samples stored at 12 °C had deteriorated below the limit of acceptability after opening the packages, even though it appeared to be acceptable before opening.

Additional discussion

Storage temperature is known to be the single most important factor influencing the quality of fresh-cut products, with low temperature well documented as beneficial for quality maintenance (Smyth and others 1998; Jacxsens and others 2002; Kader 2002; Izumi and others 2005). In general, low temperature decreases the respiration rate and enzyme activity of fresh-cut product and therefore slows down physiological deterioration. Reducing temperature also reduces the growth of microorganisms and the microbial related spoilage of products. A storage temperature of 1 to 3 °C is often recommended by the fresh-cut product processors to maintain quality and prolong shelf life. However, the effect of storage temperature on fresh-cut produce safety is multifaceted. On one hand, it is well documented that increasing storage temperature can significantly increase *E. coli* O157:H7 growth, and thus increase food safety risks. On the other hand, fresh-cut produce can harbor diverse native microorganisms, and temperature abuse may increase the growth of those microorganisms, and their competition with *E. coli* O157:H7 for nutrients and space (Cooley and others 2006). Furthermore, temperature abuse can also accelerate the growth of spoilage microorganisms, and deterioration of product quality, and thus discourage consumers from eating contaminated fresh-cut products.

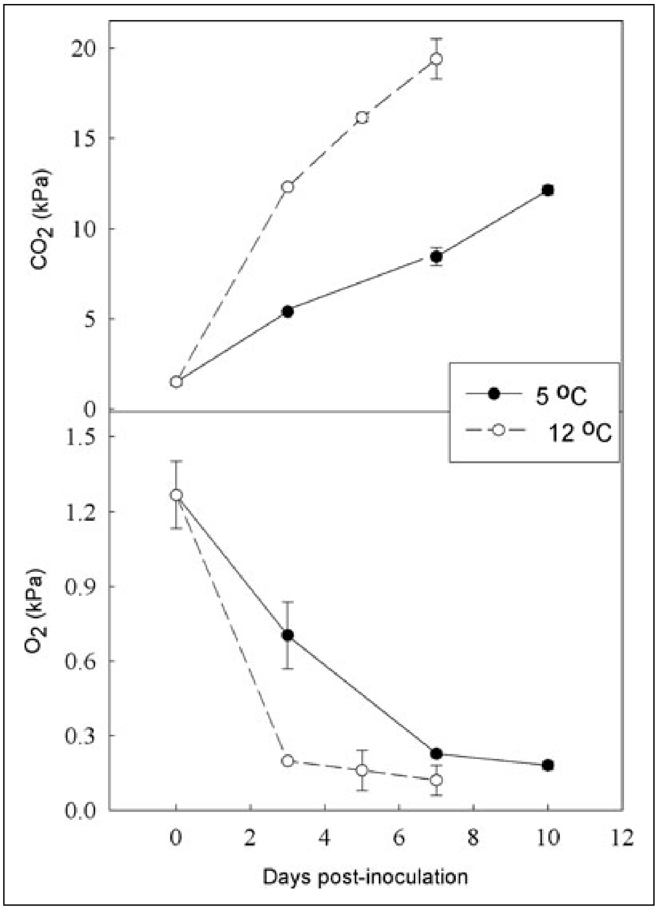

Figure 4–Trial 3-changes in CO_2 and O_2 concentrations of packaged fresh-cut lettuce stored at 5 and 12 °C, for up to 10 d. Values are the means of 4 replications.

Studies on pathogen growth of fresh-cut tomatoes and melons have shown that pathogens grow rapidly at elevated temperatures (FDA 2006). FDA uniform food code therefore includes these fresh-cut fruits in "time/temperature control for safety food" categories, and requires refrigeration at 5 °C or below (FDA 2007). However, studies exploring the effect of temperature on the safety of packaged leafy green products are scarce. Limited studies have shown that *E. coli* and *L. monocytogenes* can grow rapidly on lettuce when held at 12 °C or above (Koseki and Isobe 2005; O'Beirne 2007; McEvoy and others 2008). However, there is no scientific information regarding the quality of fresh-cut products at the time when pathogen growth is significant. Additionally, most studies have used laboratory-prepared greens, which are not washed in recycled wash water and are packaged under different atmospheric conditions and therefore may have different natural micro flora from commercially packaged products. Our previous studies have shown that *E. coli* O157:H7 can grow significantly on commercially packaged baby spinach leaves held at 8 °C or above (Luo and others 2009). However, baby spinach leaves were packaged under micro perforated film, and the behaviors of *E. coli* O157:H7 on commercially processed lettuce salad held under modified atmosphere packaging (MAP) conditions were unknown. Furthermore, leafy green vegetables grow in the natural environment with diverse temporal, spacial, and agricultural management conditions and harvesting and fresh-cut processing practices vary largely among different companies and even among different batches from the same processors. Therefore, questions were raised as to whether *E. coli* O157:H7 present on different batches of product or those grown, processed, and packaged by different firms under different conditions would have differing response to temperature abuse. This series of studies demonstrates not only that when subjected to temperature abuse, *E. coli* O157:H7 can grow significantly on commercially packaged salads of various brands, but also, that this can occur while the product appears fully acceptable for human consumption.

Delaquis and others (2007) reviewed the fate of *E. coli* O157:H7 under different atmospheric conditions and found that gas composition has no direct effect on its growth. However, there are several indirect ways in which gas composition may affect growth of *E. coli* O157:H7 (O'Beirne and Zagory 2009). First, optimal MAP can reduce produce quality deterioration, and delay the growth of spoilage organisms, and thus reduce the competition between indigenous microorganisms and *E. coli* O157:H7, leading to enhanced *E. coli* O157:H7 growth (Heard 2002; Toivonen and others 2009). Second, by reducing produce respiration rate, delaying senescence, and extending the shelf life of packaged fresh-cut salads (Francis and O'Beirne 1999), MAP may increase the time available for pathogens to grow to significant numbers, before the product begins to show overt signs of spoilage (O'Beirne and Zagory 2009; Oms-Oliu and others 2009). In this study, the commercially packaged salads maintained high visual quality scores (Figure 1 and 2) after 3 d of storage, despite the presence of significant numbers of *E. coli* O157:H7 (Figure 5). Although *E. coli* O157:H7 has a low infectious dose, the risk and severity of illness increases with the number of cells consumed (Teunis and others 2004; Strachana and others 2005). These factors suggest the importance of temperature control to ensure the safety of commercially packaged vegetable salads during processing, distribution, and storage, as abusive temperatures can facilitate the rapid proliferation of *E. coli* O157:H7, prior to product quality deterioration.

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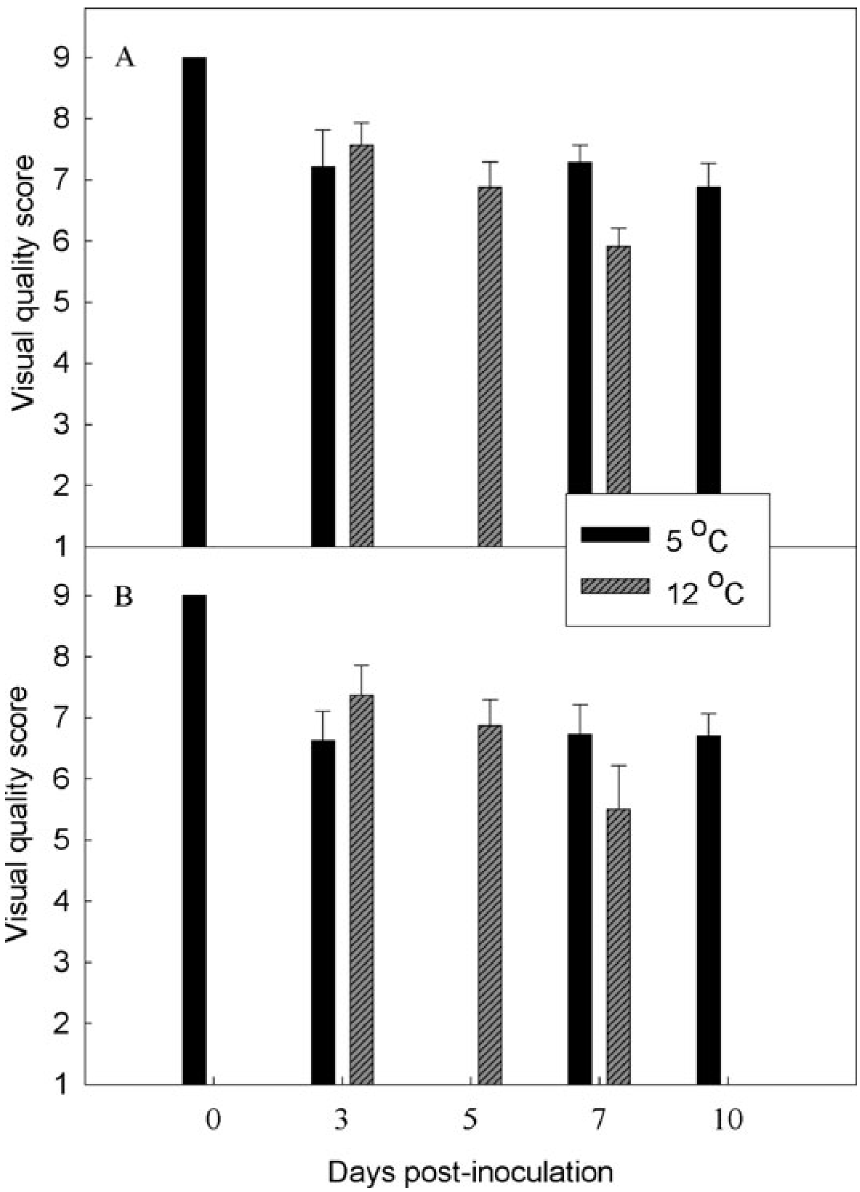

Figure 5–Trial 3-visual quality of lettuce stored at 5 and 12 °C for up to 10 d (B) before and (A) after opening the package. 1 = extremely poor, not acceptable; 5 = lower limit of sales appeal, slightly to moderately objectionable defects; 9 = excellent, essentially free from defects. The vertical bars represent the standard errors of the means of 4 replications. Samples stored at 12 °C were tested more frequently, with a shorter duration, than those stored at 5 °C.

Conclusions

This study demonstrated that *E. coli* O157:H7, strain F6460, can grow significantly on commercially packaged fresh-cut salad containing Romaine and Iceberg lettuce when held at 12 °C. Although holding products at this abusive temperature also accelerates product quality deterioration, significant growth of *E. coli* O157:H7 can occur prior to the overt signs of product quality deterioration. Additionally, there is a significant die-off of *E. coli* O157:H7 when the products are stored at 5 °C. This study underlines the importance of temperature control in supply chain to ensure the safety of commercially packaged fresh-cut leafy green vegetables.

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